Circular dichroism measurements show that C·C⁺ base pairs can coexist with A·T base pairs between antiparallel strands of an oligodeoxynucleotide double-helix

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ABSTRACT

We have studied the coil-to-helix transition of the DNA oligomer d(C₄A₄T₄C₄), using circular dichroism measurements to monitor the formation of A·T base pairs within the central self-complementary A₄T₄ region and the formation of protonated C·C⁺ base pairs at the ends of the oligomer. We found that both A·T and C·C⁺ base pairs formed in a coordinated fashion as the temperature and pH were lowered. The CD data of the helix form of the oligomer were consistent with the presence of paired oligomers, but not with hairpin loops. The pK_a for formation of C·C⁺ base pairs between the C₄ ends of the oligomer was higher than the pK_a for formation of C·C⁺ base pairs in d(C₈), indicating that the formation of C·C⁺ base pairs in the oligomer was influenced by the presence of a paired A₄T₄ region. We conclude that A·T and C·C⁺ base pairs coexist in the self-complex of the oligomer and, therefore, that C·C⁺ base pairs can form between antiparallel DNA strands.

INTRODUCTION

Helical forms of poly[r(C)], poly[d(C)], and poly[d(C-T)] are stabilized by protonated cytosine base pairs.¹⁻⁶ For each of these polymers, the formation of a helical complex requires the addition of one proton per two cytosine bases.^{3,4,6} Figure 1(A) shows the hydrogen bonding for a hemiprotonated pair of cytosines (C·C⁺ base pair) as proposed by Langridge and Rich¹ from their fiber diffraction study of an acid form of poly[r(C)]. In this scheme, one added proton is shared between the N3 ring nitrogens of two cytosines, which are thus stabilized by three hydrogen bonds. The symmetry of this base pairing requires either that the paired polynucleotide strands be parallel, as was proposed for poly[r(C)] in fibers,¹ or that the base-sugar conformations of the paired nucleotides differ, one being *anti* and the other being *syn*. In subsequent work, Arnott *et al.*⁷ showed that the fiber diffraction pattern obtained by Langridge and Rich¹ actually resulted from a stacked, single-stranded structure of poly[r(C)], leaving open the question as to the type of base pairing that would exist in the acid duplex form of poly[r(C)]. Base pairing of the type shown in Figure 1(A) does exist in the crystal structure

of cytosine-5-acetic acid,⁸ and it also exists between the cytosines of a parallel dimer of CpA co-crystallized with proflavine.⁹ Moreover, there is steric hindrance to rotation about the glycosidic bond in pyrimidine-containing nucleotides,¹⁰ so that the *syn* conformation of cytidyl residues is disfavored. Thus, although there is no direct evidence that the helical forms of poly[r(C)], poly[d(C)], and poly[d(C-T)] contain base pairing of the type shown in Figure 1(A) between parallel strands, it has been reasonable to assume that they do.²⁻⁶ If this were the only type of pairing possible between cytosines, C·C⁺ base pairs could not form between antiparallel strands of a natural DNA or between antiparallel regions within a hairpin loop of a single strand of DNA.

We have tested the possibility that C·C⁺ base pairs might form between antiparallel strands of DNA, using CD measurements to distinguish stacked C·C⁺ base pairs from stacked A·T base pairs in the DNA sequence d(C₄A₄T₄C₄). If C·C⁺ base pairs can only form between parallel strands of this oligomer, then helical forms of the oligomer will involve only one of the two types of base pair, to give either a central region of A·T base pairs between antiparallel strands, sketched as structure I,

or to give C·C⁺ base pairs between the ends of parallel strands, sketched as structure II,

However, if C·C⁺ base pairs can form between antiparallel strands of the oligomer, both types of base pair can exist in the same helical form, sketched as structure III,

Figure 1. (A) The type of C·C⁺ base pair that was proposed by Langridge and Rich¹ for the acid helical form of poly[r(C)]. (B) A type of C·C⁺ base pair that can be accommodated between the antiparallel strands of a Watson-Crick double helix without requiring the unfavorable syn conformation in one of the strands.

Our CD study provides evidence that the type III structure results from the coil-to-helix transition of the oligomer and that C·C⁺ base pairs can indeed form between antiparallel DNA strands.

MATERIALS AND METHODS

Oligodeoxynucleotides

Two DNA oligomers, d[C(pC)₃(pA)₄(pT)₄(pC)₄] (which is denoted d(C₄A₄T₄C₄) or the "16-mer") and d[A(pA)₃(pT)₄] (which is denoted d(A₄T₄)) were synthesized by the phosphite triester method on a silica gel support using a DNA synthesis kit (#1530) from New England Biolabs, Inc. The oligomers were purified by electrophoresis in 20% polyacrylamide gels containing 7 M urea, as described by Frank and Koster.¹¹ Oligomer bands were visualized in a gel by their ability to quench a fluorescence indicator in a thin layer chromatography plate, placed beneath the gel, upon illumination with UV light. A series of bands corresponding to different oligomer lengths, plus a band of low mobility presumably due to oligomers still bound to silica gel, were observed from the synthesis mixture of each oligomer. The oligomer bands of highest molecular weight, which had the mobilities expected for d(C₄A₄T₄C₄) and d(A₄T₄) in comparison with standards, were cut from the gels and eluted in 0.5 M ammonium acetate and 1 mM disodium EDTA (pH 8.0) as described by Maniatis *et al.*¹² The eluate was concentrated to about 1 ml in vacuum and, after a one-fifth volume of 1.47 M sodium acetate was added, the oligomers were precipitated by adding 4 volumes of ethanol. The precipitate was allowed to form at -20°C or -70°C for about 1 hour and was collected by centrifugation

for 30 min at 10,000 rpm (12,000g max) in a Sorvall SS-34 rotor at 4°C. After drying in a vacuum, the oligomers were dissolved in 0.01 M Na⁺ (phosphate buffer, pH 7.0), and a sufficient volume of a 4 M NaCl solution was added to raise the concentration of Na⁺ to 0.5 M. The residual acetate ion and urea concentrations in solutions of the oligomers used for spectral measurements were estimated to be less than 1 mM and 2 mM, respectively. The pH was adjusted to 6 or 5 by the addition of H₃PO₄. Reported pH values are accurate to ± 0.1 pH unit.

The lyophilized sodium salt of the DNA oligomer d[(pC)₈] (which will be denoted as d(C₈)) was purchased from Pharmacia P-L Biochemicals, Inc., and was used without further purification. This oligomer was also dissolved in 0.01 M Na⁺ (phosphate buffer, pH 7.0), and the Na⁺ concentration and the pH were adjusted as described above for the two oligomers we synthesized.

CD Measurements

Circular dichroism measurements were made using a Cary Model 61 circular dichrometer, calibrated as in previous work.¹³ Sample temperatures were controlled thermoelectrically to ± 0.5°C. Spectra were smoothed by a sliding 13-point quadratic (or cubic) function¹⁴ and were plotted on a Hewlett-Packard 7470A plotter. Concentrations were determined by absorbance measurements and the following extinction coefficients: 10,390, 7,260, and 8,700 liter·mol⁻¹·cm⁻¹ for d(A₄T₄), d(C₈), and the 16-mer, respectively. These extinction coefficients are first-neighbor estimates calculated from the known extinction coefficients of dimers and monomers.¹⁵

Estimates of the CD of the 16-mer

Averages of the CD of d(A₄T₄) and d(C₈) were used to estimate the CD of the 16-mer under given conditions. If the conformations of all three oligomers are identical in their helical or single-stranded (coil) forms, such an estimate is exact for all of the monomer CD contributions and for 13 of the 15 first-neighbor CD contributions in the 16-mer. Only the CpA and TpC first-neighbors of the 16-mer do not exist in the d(A₄T₄) or d(C₈) oligomers.

RESULTS

Model DNA Oligomers

Two DNA oligomers were used as models to obtain the CD contributions of the regions of the 16-mer that might form A·T and C·C⁺ base pairs. The first of these was d(A₄T₄), corresponding to the central self-complementary sequence of the 16-mer. CD spectra of this compound are shown at several temperatures and pH values in Figure 2. Spectra of the helix form at 0°C and of

Figure 2. CD spectra of helix (0°C) and coil (40°C) forms of the model oligomer $d(A_4T_4)$ at pH 5 and 7. The nucleotide concentration was 7.1×10^{-5} M. Inset: melting profiles of the oligomer at pH 5 and 7. The oligodeoxynucleotide solutions used to obtain the CD data in this and subsequent figures all contained 0.5 M Na^+ .

the coil form at 40°C were only slightly affected by pH over the range of 5 to 7. Melting profiles at pH 5 and 7 were similar, as shown in the inset in Figure 2. A major difference between the CD spectra of the helix and coil forms of $d(A_4T_4)$ was that the helix form had an enhanced negative band at about 250 nm. Differences above 260 nm are also apparent in Figure 2, but these were not as useful in distinguishing A·T from C·C⁺ base pairs since the formation of C·C⁺ base pairs resulted in much larger CD changes above 260 nm. The appearance of a negative CD band near 250 nm is also a characteristic of the coil-to-helix transitions of poly[d(A)·d(T)] and poly[d(A-T)·d(A-T)].¹⁶

There is little doubt that we obtained the duplex form, and not a hairpin loop, of the $d(A_4T_4)$ oligomer at 0°C and a Na^+ concentration of 0.5 M, with a nucleotide concentration of 7.1×10^{-5} M. Duplex and hairpin loop forms of a series of (AT)_n oligomers have been well-characterized by Scheffler *et*

Figure 3. CD spectra of the model oligomer $d(C_8)$ at pH 7, 0 and 20°C, in its coil form. The nucleotide concentration was 1.0×10^{-4} M for the spectra in Figures 3 and 4.

al.^{17,18} These oligomers, at optical concentrations, do not form hairpin loops below 40°C at a Na^+ concentration of 0.51 M, except as a metastable state.¹⁸ Moreover, a short oligomer like $d(AT)_5$ has a strong preference for duplex formation below 40°C at 0.51 M Na^+ because a hairpin loop in this oligomer can be closed by only three (or at most four) base pairs.¹⁸ Our $d(A_4T_4)$ oligomer would be even less likely to have formed a hairpin under similar conditions since it could be closed by only two (or at most three) base pairs.

The second model compound that we used was $d(C_8)$. The CD spectra of $d(C_8)$ are shown at several different temperatures and pH values in Figures 3 and 4. As seen in Figure 3, there was no evidence of a coil-to-helix transition at pH 7 when the temperature was lowered to 0°C. The spectra in Figure 3 are typical of the single-stranded form of poly[d(C)].^{19,20} At pH values of 6 and 5, however, large CD changes occurred for the $d(C_8)$ oligomer when the tem-

Figure 4. CD spectra of the model oligomer $d(C_8)$ in its coil form at pH 6, 40°C, and with increasing amounts of helix form at pH 6, 0°C, and pH 5, 0°C. Inset: CD melting profiles of the oligomer at pH 5 and 6.

perature was decreased to 0°C. As shown in Figure 4, a positive band appeared at 285–290 nm and a negative band appeared at 260–265 nm in the spectrum of $d(C_8)$ as the temperature was decreased at these lower pH values. These bands undoubtedly represented the helical duplex form of the oligomer, containing stacked $C \cdot C^+$ base pairs, since identical bands appear in the spectrum of the self-complex of poly[d(C)].^{19,20} (The $d(C_8)$ oligomer, like $d(A_4T_4)$, should not form a hairpin loop under our solution conditions.) There was minimal change in the CD at 250 nm, where a maximal CD change occurred upon formation of A·T base pairs in A_4T_4 . Thus, the formation of $C \cdot C^+$ and A·T base pairs can be easily distinguished by their CD characteristics.

The only difference between the coil-to-helix transitions of $d(C_8)$ and poly[d(C)] is that the protonated helical form of poly[d(C)] occurs at neutral pH and 20°C,⁴ while lower pH and temperature were required for formation of the protonated self-complex of the $d(C_8)$ oligomer. As seen in the inset to

Figure 5. CD spectra of the 16-mer $d(C_4A_4T_4C_4)$ in its coil form at pH 7 (20°C), pH 6 (40°C), and pH 5 (40°C). The calculated averages of the CD spectra of the model oligomers $d(A_4T_4)$ and $d(C_8)$ under the same conditions are shown for comparison. The nucleotide concentration was 6.5×10^{-5} for the spectra in Figures 5 through 10.

Figure 4, the CD change did not quite reach a plateau even at pH 5 and 0°C. The difference in the pK_a values for the conformational transitions of $d(C_8)$ and poly[d(C)] is due to the greater stability of the polymer relative to the oligomer, resulting in a larger shift from the intrinsic pK_a of deoxycytidine (which is 4.3²¹).

The 16-mer

CD spectra of the 16-mer in the single-stranded (coil) form at different pH values are shown in Figure 5. At temperatures of 20°C (pH 7) and 40°C (pH 5 and 6), the spectra of the 16-mer were quite similar and were in agreement with the averages of the spectra of $d(A_4T_4)$ and $d(C_8)$ under the same conditions. Except for $d(A_4T_4)$ at pH 7 and 20°C (Figure 2, inset), neither $d(A_4T_4)$ nor $d(C_8)$ showed any evidence for formation of base pairs under these conditions, and, therefore, the spectra shown in Figure 5 are essentially

Figure 6. CD spectra of the 16-mer at pH 7, 0°C (—) and 20°C (- - -). The average of the spectra of the model oligomers at pH 7, 0°C (x x x).

those of the single-stranded form of the 16-mer.

Figures 6, 7, and 8 show the changes in the CD spectrum of the 16-mer that occurred at the three pH values of 7, 6, and 5 as the temperature was lowered to 0°C. At all three pH values, a negative band at 250 nm, a negative band at about 265 nm and a long-wavelength positive band above 280 nm appeared as the temperature was decreased to 0°C. These CD features were those found during the coil-to-helix transitions of d(A₄T₄) (the 250 nm band) and d(C₈) (the 265 and 285 nm bands), described above. The calculated averages of the spectra of the model oligomers at 0°C are shown in Figures 6, 7, and 8 for comparison with the measured spectra of the 16-mer at 0°C. The calculated spectra included all the CD changes that occurred during the coil-to-helix transitions of both model oligomers. There was good agreement between the magnitudes of the measured and calculated 250 nm bands in all three cases, implying that similar conditions were needed for formation of A·T base pairs

Figure 7. CD spectra of the 16-mer at pH 6, 0°C (—) and 40°C (- - -). The average of the spectra of the model oligomers at pH 6, 0°C (x x x).

in the 16-mer and in $d(A_4T_4)$. The appearance of CD bands in the 265 and 285 nm regions indicated the formation of $C \cdot C^+$ base pairs in the 16-mer, especially at the lower pH values. However, there was an interesting difference between the measured and calculated magnitudes of the 265 and 285 nm bands. The magnitudes of these bands in the spectra of the 16-mer at all three pH values, 0°C, were enhanced relative to these bands in the calculated spectra and, hence, in the spectra of $d(C_8)$ taken under the same conditions. Even at pH 7, the CD spectrum of the 16-mer at 0°C showed evidence of the formation of $C \cdot C^+$ base pairs, although the $d(C_8)$ oligomer was single-stranded under these conditions. Thus, the presence of a complementary A_4T_4 sequence in the center of the 16-mer increased the pK_a for $C \cdot C^+$ base pair formation in the 16-mer, relative to $d(C_8)$. This would not be expected if $C \cdot C^+$ base pairs could form only between the ends of parallel 16-mers as in the type II structure described in the Introduction, since two regions of 4 $C \cdot C^+$ base pairs each (containing

Figure 8. CD spectra of the 16-mer at pH 5, 0°C (—) and 40°C (- - -). The average of the spectra of the model oligomers at pH 5, 0°C (x x x).

a total of 6 nearest-neighbor base pair stacks) should be less stable and have a lower pK_a than the one region of 8 base pairs containing 7 nearest-neighbor stacks in paired $d(C_8)$ oligomers. The increased pK_a for formation of $C \cdot C^+$ base pairs in the 16-mer would be expected if they were stabilized by adjacent A-T base pairs, as in the type III structure.

The extent of $C \cdot C^+$ base pair formation in the 16-mer at pH 6, 0°C, was very similar to that in the $d(C_8)$ oligomer at a lower pH, as shown by the agreement in Figure 9 of the measured CD spectrum of the 16-mer at pH 6, 0°C, and the calculated average of the spectra of the model oligomers at pH 5, 0°C. The intensities of the 265 and 285 nm bands actually observed for the 16-mer at pH 5, 0°C, were greater than those found for $d(C_8)$ under the same conditions (Figure 8), as pointed out above, and presumably corresponded to an even more complete base pairing of the cytosines in the 16-mer than in $d(C_8)$ under these conditions.

Figure 9. CD spectrum of the 16-mer at pH 6, 0°C (—), compared with the average of the spectra of the model oligomers at pH 5, 0°C (x x x).

The coordinated formation of A·T and C·C⁺ base pairs in the 16-mer is demonstrated by the data in Figure 10, which show that the negative CD band at 250 nm and the positive band at 285 nm (indicative of A·T and C·C⁺ base pairs, respectively) simultaneously appeared as the temperature was decreased at pH values of 6 or 5.

DISCUSSION

As outlined in the Introduction, if C·C⁺ base pairs could only form between parallel DNA strands we would not expect to have both A·T and C·C⁺ types of base pair form in the 16-mer under the same solution conditions. Not only did both types of base pair form in the 16-mer in a coordinated fashion as the temperature and pH were varied, but the pK_a for formation of C·C⁺ base pairs was increased in the 16-mer relative to the d(C₈) oligomer, presumably because the stability of C·C⁺ base pairs was enhanced by the neighboring self-comple-

Figure 10. CD versus temperature for the 16-mer at (A) pH 6 and (B) pH 5. A negative band at 250 nm and a positive band at 285 nm simultaneously appear at lower temperatures; these bands indicate the formation of A·T and C·C⁺ base pairs, respectively, in the 16-mer double helix.

mentary A₄T₄ sequence in the 16-mer. Thus, our data are not consistent with the existence of a simple mixture of structures of types I and II. A higher order structure that involved both type I and II structures in a complex stabilized by interacting parallel and antiparallel duplexes might be envisaged to explain our data. However, the close agreement of the CD spectra of the 16-mer with the CD spectra of simple model compounds lends strong support to the straightforward model that base pairs form between antiparallel strands of the 16-mer, as in structure III.

Among alternative structures that we believe may be excluded for the self-complex of the 16-mer are those involving pairing between protonated adenines; the formation of the acid structure of poly[d(A)] requires a pH of less than 4.5 (at 0.1 M NaCl),²² well below the pH needed for formation of the self-complex of the 16-mer.

It is possible that C·C⁺ base pairs could form within a hairpin loop of a single 16-mer molecule. However, data on other DNA oligomers similar in length show that hairpin loop formation is not favored at a Na⁺ concentration of 0.5 M, given the temperatures and the oligonucleotide concentration we used.^{17,18,23} Moreover, we have no evidence for a biphasic melting profile and, in a separate experiment, we found that the CD melting profile of the 16-mer at pH 5 was shifted to lower temperatures by at least 2°C upon a 5-fold dilution in nucleotide concentration from 6.5 × 10⁻⁵ to 1.3 × 10⁻⁵ M (data not shown), which is not consistent with hairpin loop formation. Also, intramo-

lecular base pairing would require a loop of probably 4 unpaired bases¹⁸ in the central A₄T₄ region of the 16-mer. This does not seem likely in view of the generally excellent agreement shown in Figures 6, 7, and 8 between the measured magnitude of the 250 nm band in spectra of the helix form of the 16-mer and the calculated magnitude of this band, obtained from d(A₄T₄) in the duplex form. Only in the case of the 16-mer at pH 5 and 0°C was this band less intense than was calculated (Figure 8). It is possible that the slight decrease in the 250 nm band in this case was due to some destabilization of the A·T base pairs at the junction with fully hemiprotonated C·C⁺ base pairs. Finally, even if the C·C⁺ base pairs we detected in the 16-mer did form between the ends of a hairpin loop, this would not affect our major conclusion that such base pairs can form between antiparallel strands, since the ends of a hairpin loop are antiparallel.

What is the arrangement of cytosines in the C·C⁺ base pairs that form between antiparallel strands of the 16-mer? If the bases are paired as shown in Figure 1(A), then one of the sugar-base angles must be syn, while the other is anti. A syn conformation of the glycosidic bond is sterically hindered in pyrimidine nucleosides,¹⁰ although the syn form can be imposed on a pyrimidine deoxynucleoside by a bulky methyl group at position C6 of the base, as shown by the crystal structure of 6-methyl-2'-deoxyuridine.²⁴ Moreover, we have found it difficult to construct a double helix containing cytidyl residues paired as shown in Figure 1(A), with one syn and one anti glycosidic bond, in juxtaposition to normal Watson-Crick base pairs in a space-filling model of B-DNA. Therefore, we believe that the cytosines in the helix form of the 16-mer may not be paired by the scheme shown in Figure 1(A). An alternative C·C⁺ base pairing arrangement is shown in Figure 1(B). In this arrangement, which also requires one proton per two cytosine bases, the cytosines are asymmetrically paired with two hydrogen bonds, one between a protonated N3 donor and a C2-O acceptor and another between an unprotonated N3 acceptor and a C4-N donor. Cytidyl residues paired in this manner, and with anti glycosidic bonds in both strands, can be reasonably stacked against a neighboring Watson-Crick base pair in a model of B-DNA. Two isomeric stacking arrangements are possible, depending upon which N3 is protonated.

Whatever the type of base pairing, our finding that C·C⁺ base pairs can form between antiparallel strands of the DNA oligomer d(C₄A₄T₄C₄) raises the possibility that the strands in the helical form of the d(C₈) oligomer, as well as the strands of the helical forms of poly[r(C)], poly[d(C)] and poly[d(C-T)], may also be antiparallel, at least under some conditions. The

close agreement of the CD bands in the helix form of $d(C_8)$ and those attributed to the $C \cdot C^+$ base pairs in the 16-mer (see Figure 9) indicates that the base stacking and probably the base pairing are similar in these two cases.

The knowledge that cytidyl residues can pair between antiparallel strands of DNA increases the recognized repertoire of structures available to nucleic acids. Polypyrimidine-polypurine segments are ubiquitous in the DNA of higher organisms,²⁵ and some of these are cytosine-rich, such as the *Drosophila virilis* DNA sequence consisting of a $(C-T)_{8-18}(C)_{4-5}$ repeat for about 400 base pairs²⁶ and the sequence T-C-C-C-T-C-C-C-T-T-C-C-T-C-C-T-C-C-C-T, which flanks the 5' end of the chicken pro- $\alpha 2(I)$ collagen gene.²⁷ Tracts of 100-150 nucleotides containing over 75% C have been found in the RNA genomes of encephalomyocarditis virus and several other cardioviruses.²⁸ Such cytosine-rich strands could fold back on themselves to form structures stabilized by $C \cdot C^+$ base pairs. It is likely that $C \cdot C^+$ base pairs can also form in $d(G-C)_n$ sequences,^{5,6,29} whose diverse conformational range includes the left-handed Z-DNA conformation.³⁰ Structures stabilized by $C \cdot C^+$ base pairs, if they can form in natural DNA and RNA sequences, may participate in sequence-dependent control functions.

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